Ethanol from Biomass
A Genetic Engineering and Microbial Approach

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CONTENTS

Abstract

Cellulases were evaluated for consideration of the efficient hydrolysis of

cellulose to glucose. Trichoderma reesei was immobilized on Celite and using

lactose as a substrate/inducer and gave yields of up to 200 fpu/l/hr.

Concentrations were low (2 fpu/ml). Good enzyme productivity was maintained

over 2-4 weeks. Decreasing carbon: nitrogen ratios of the nutrient resulted in

major sporulation in submerged phase. N-Deglycosylation of T. reesei did not

give any change in pH optima or pH stability of the cellulase. An efficient thermally

stable cellulase obtained from an actinomycete, Microbispora bispora, was

purified and shown to contain cellobiohydrolases, endoglucanases and beta-glucosidases

which acted together synergistically. This synergistic mechanism, well known

in fungi, was clearly shown to present in this bacterium. Initial characterization

of an endoglucanase from Pseudomonas fluorescens via cloning was accomplished.

The development of a basic genetic system for studying the ethanologen was

addressed. Following insertion of the lac (beta-galactosidase) operon into

Z. mobilis, subsequent mutation failed to raise beta-galactosidase levels.

In order to gain enhanced enzyme production, fundamental genetic protocols were

considered including the isolation of Zymomonas origins of replication and

promoters. Studies on developing a Zymomonas transformation system were

partially successful. Antibiotic sensitive mutants were isolated and have

proved useful in facilitating plasmid transfer to Zymomonas based on selection

of inheritance of antibiotic resistance.

Introduction

Cellulose, a major component of all vegetation, is one of the world's most plentiful energy resources. Unlike other resources, such as fossil fuels, cellulose is constantly replenished via photosynthesis, with estimates of total fixation of about 2×10^{11} metric tons of carbon per year (Hall, 1980). This represents ten times the world's annual energy use and two hundred times our direct food energy consumption (Hall, 1980). A practical evaluation of biomass in the U.S.A. assesses yields from forest products (600×10^6), agriculture residues (550×10^6 tons) and cellulosic municipal garbage (160×10^6 tons) to total 1.3 billion tons/annum (Goldstein, 1981). By use of a further "practicality" factor of 30%, it is predicted that 400 million tons of biomass are available per year, and thus could provide an adequate base for the United States' annual usage of 40 million tons of chemical and polymers plus the 60 billion tons of fossil hydrocarbon feedstocks (Goldstein, 1980). On this basis a scenario can be drawn in which cellulose can serve as a feedstock for the fermentative preparation of alternate fuels (ethanol, methane), chemicals (acetic, acrylic and citric acids, butanol) and foodstuffs (lysine, monosodium glutamate, single cell protein) (Bungay, 1981; Eveleigh, 1981; Goldstein, 1981; Ng et al., 1983). This project is based on schemes to convert cellulose enzymatically (via cellulases) to glucose as a fermentation substrate for the preparation of chemical feedstocks. Alcohol production by the ethanologen _Zymomonas mobilis_ is considered as one example of feed stock production. The report is divided into four sections:

    I.   _Trichoderma reesei_ cellulase

   II.   _Microbispora bispora_ cellulase

  III.   Other Cellulases

   IV.   Developmental genetics of _Zymomonas mobilis_

I. Trichoderma Cellulase: Enzymatic conversion of cellulose to glucose is

under wide study (Finland, India, Japan, South Africa, Sweden, USA - see Fiechter,

1982; Ghoshe, 1980; RAPAD, 1982; Scott, 1982). The wide adoption of this methodology

probably is influenced by the fact that the final stage for production of chemicals

is via fermentation. The conversion efficiency of Trichoderma reesei cellulase

enzymes is extremely high with 98% saccharifications having been reported.

Glucose syrups produced by enzymatic hydrolysis, lack the presence of inhibitory

by-products, and are readily fermented. Unfortunately, the cost of cellulase

production is high, and thus a variety of hypercellulolytic mutants have been

selected especially at Rutgers to circumvent this problem (Cuskey et al., 1983).

Consideration of enhanced cellulase production using cells immobilized on

Celite has been one research task. Other tasks included spore production

combined with cellulase production, and also the role of glycosylation of

cellulases.

I.A. Immobilization of Trichoderma reesei for Cellulase Production (E. Frein)

IA (1). Cellulase Production: Cellulase production by the immobilized T.

reesei culture was evaluated via immobilization of the cells on Celite (Frein

et al., 1983). Recent productivities averaged 175 FPU/L/hr and even reached 270

FPU/L/hr (Fig. 1). Support loading (g cell/ g Celite) was as high as 2.1; this

figure represents a cell loading of 107 g cell/L fermentor (Fig. 1B). Average

loading were around 50 g/L. Reduction in carbon supply perturbed the steady

state population and this was reflected in lowered cell loadings, and transiently

higher cellulase productivities possibly due to cell death and lysis (Fig. 1A &

B). Interestingly, the magnitude of this response seems to be independent of

the magnitude of carbon source reduction; the response is repeatable provided

cell loadings do not fall below 0.25 g cell/g Celite. This suggests that a

fermentation in which immobilized Trichoderma is cycled through periods of

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carbon starvation, can result in higher overall productivities than a similar steady-state fermentation.

Thus good yields of cellulase have been obtained through the use of cells immobilized as Celite. However, though Celite is inexpensive, nevertheless its use adds to fermentation costs and this must be factored into the economics of cellulase production. As an alternative route to maintaining high cell densities, mycelial pellets can be used in place of Celite immobilized cells. In order to investigate this aspect, an airlift fermentor was inoculated with spores of T. reesei (5 x 10^6/ml) and run in a batch mode until mycelial pellets were formed (7 days.) The fermentation wan then shifted to a continuous mode of operation and culture continued for 25 days.
The mycelial pellets gradually became quite large (5-8 mm diameter.) This size allowed efficient retention through use of a simple filter on the outlet port. In comparison with Celite-immobilized cells, this fermentation was troublefree; e.g. the outlet screen rarely becoming clogged. Cellulase production by the fungal pellets reached 63 FPU/L/hr and averaged 50 FPU/L/hr for 25 days. This is lower than the 200 FPU/L/hr productivities achieved with Celite-immobilized T. reesei, and is probably due to both lower cell concentration and also poorer mass transport to and from the center of these large pellets. These observations are borne out in that the cell loading (70 g/l) was not as great as a Celite fermentation (100 g/l) and that specific cellulase productivity is also less than that of a "Celite" fermentation.

IA (2). Sporulation: The production of spores by Trichoderma is of possible industrial significance due to their potential as a biocontrol agent of plant pathogens (Eveleigh, 1985). The simultaneous production of both spores and cellulase by T. reesei is possible, and may provide an economic approach to cellulase production since the spores represent an additional

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marketable product and are easily separated and recovered from the cellulase fermentation.

Studies were made to assess the feasibility of concurrent spore production during a cellulase fermentation. Sporulation is not a well-understood phenomenon, especially as regards induction and its physiological causes. In the case of T. reesei sporulation has been attributed to the effects of light (Jensen, 1971) and additionally to physical contact and nutritional status. Our previous experience with immobilized T. reesei RUT C_{30} in carbon-limited continuous culture, led us to suspect that sporulation could be induced by carbon-limitation. On several occasions this had apparently occurred and as a result we decided to test this hypothesis.

T. reesei spores were "immobilized", as detailed previously (Frein et al., 1983) and allowed to germinate in batch culture and thence to continuous culture. A descending lactose gradient was used to induce carbon limitation (starvation). Morphologically the hyphae were thinner, larger and less branched. Large thick-walled chlamydospores formed in intercalary positions. Sporulation (philalospores) were not induced. However, our initial hypothesis was not supported as sporulation of immobilized T. reesei was not induced by carbon limitation.

Subsequently a nitrogen-limited fermentation was examined for both its ability to induce sporulation and also to determine the levels of cellulase produced. A Celite-immobilized culture of T. reesei was developed as previously reported, and once steady-state was reached, subjected to a stepwise decreasing C:N ratio from 8:1 (normal growth / cellulase prod medium) to 22:1, the amount of carbon (0.34 lactose) was kept constant. Sporulation as determined by the formation of spore-bearing structures (phialides limited to hyphal tips) were initiated at a C:N ration of 10:1 and was markedly and highly apparent at a C:N ration of 12:1. Spore counts reached 2 x 10^7/mL effluent.

It is notable that this level of spore production represents 2×10^9 spores/L fermentor/hr and that this level was maintained for eighteen days.

Obviously, nitrogen limitation is not conducive to either cell growth or "protein" production. Indeed, cell loading (g cell/g support) obtained in this experiment were lower than those obtained through carbon limitation at the level used here (0.3%) - 0.3 g cell/g support as 1.0 g cell/gm support.

Cellulase production was similarly reduced averaging 82 FPU/L/hr, which, although not as good as obtained under optimum conditions (200 FPU/L/hr) is respectable, compared to batch operation of 25-70 RPU/L/hr.

I.A (3). <u>Glycoprotein</u> <u>Nature</u> <u>of</u> <u>Trichoderma</u> <u>reesei</u> <u>Cellulase</u> (K. Murphy-Holland)

The interest in cloning <u>Trichoderma</u> <u>reesei</u> cellulase genes into a prokaryotic vector has spurred research into the role of the oligosaccharide moieties of these glycoprotein enzymes. Routinely, prokaryotic DNA <u>in</u> <u>vivo</u> systems do not glycosylate enzymes of eukaryotic origin. As one objective is the insertion and transcription of <u>Trichoderma</u> genes in a bacterial system, it is important to know if the protein portion of cellulase is active and stable without addition of its glycosyl moieties. <u>T.</u> <u>reesei</u> cellulases contain two types of oligosaccharide-protein linkages:

1. N-linked through the amide nitrogen of asparagine.

2. O-linked through the hydroxyl group of serine or threonine.

Two approaches have been used in our laboratory to prepare cellulase enzyme deficient in N-linked oligosaccharide: first through the <u>in</u> <u>vivo</u> inhibitory action of tunicamycin to prevent glycosylation and secondly via the <u>in</u> <u>vitro</u> action of endo-beta-acetylglucosaminidase (Endo H) towards cellulase, leaving a one N-acetyglucosamine residue-asparagine attachment site on the protein and releasing the glycosyl moiety. <u>In</u> <u>vivo</u> cellulase production in the presence of TM prevents all N-linked glycosylation while endo H <u>in</u> <u>vivo</u>

treatment of an intact enzyme will cleave N-linked oligosaccharides whose cleavage sites are exposed to the attack. In the case of both TM and Endo H, only partially deglycosylated enzymes are formed in that neither treatment affects the O-linked oligosaccharides. Partially N-deglycosylated cellulase has been prepared from two strains of T. reesei, the parent QM6a, and the hypercellulolytic, catabolite repression resistant mutant, RUT-C30, using both TM and Endo H protocols.

Protein production by T. reesei QM6a is not affected by cellulase induction in the presence of tunicamycin (10 g/ml), the same amount of protein occurring in extracellular, intracellular and mycelial bound locations. For RUT-C30, less protein was secreted and greater protein remained cell bound in the TM treated cells. The specific activities of carboxymethylcellulase (CMCase) and acid-swollen cellulase (ASCase) were the same for both control and TM-treated QM6a cultures. For RUT-C30, specific activities are higher for the enzymes from the TM-treated cells. Intracellular activities are the same for both control and TM-treated RUT-C30 strains, and specific activities of mycelial bound CMCase and ASCase are higher in the control culture than in the TM-treated cells.

Non-denaturing "PAGE" electrophoresis of extracellular proteins of both TM-treated QM6a and RUT-C30 showed different protein patterns than the controls. All of the proteins reacted positively when stained for glycoprotein. Several of the TM-treated components show a slightly faster electrophoretic mobility than proteins in the control and were identified as active exosplitting-glucanases. Four other bands of slower mobility have been identified as active endoglucanases but no change in mobility was noted in control and TM-treated enzymes. In RUT-C30, four protein bands of slower mobility are present only in the control culture. These glycoproteins do not

exhibit activity against carboxymethyl cellulose, cellulo-oligosaccharides or aryl-glucosidase substrates with "in gel" assays. Isoelectric focusing indicated that all of the proteins are acidic (IEF: pH 3.9 to 5.10). To assess the role of glycosylation in temperature stability, endoglucanases from control and TM-treated QM6a and RUT-C30 were heated and residual activities compared. No differences in heat stability were observed.

Endo H cleavage of N-linked oligosaccharide of cellobiohydrolase I (CBH I) of RUT-C30 resulted in a protein with approximately the same activity as the control. The Endo H treated protein had a slightly faster electrophoretic mobility than the control enzyme and had the same specific ASCase activities.

In summary, cellulase components that have been deglycosylated via enzymatic cleavage (Endo-H treatment) or by synthesis in the presence of tunicamycin, continue to show activity equivalent to that of the fully glycosylated enzyme. No change in temperature stability of the deglycosylated enzymes was noted. These results are considered promising in relation to prokaryotic processing of T. reesei RNA and with regard to the apparent lack of a need for glycosylation in order to maintain enzyme activity. (For final report see K. Murphy-Holland, Ph.D. thesis 1985).

II. Actinomycete cellulase (Microbispora bispora)

The study of microbial cellulases was extended to include actinomycete cellulases. Sources screened focussed on generally lytic cultures available in the Rutgers Culture Collections. A variety of positive strains were isolated and their cellulases initially characterized. Microbes producing thermally stable cellulase were also screened for by selection from thermal soils obtained worldwide. An actinomycete Microbispora bispora was isolated with cellulases stable at 70°C. The beta-glucosidase of this strain was only slightly subject to end-product inhibition in comparison to Trichoderma.

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In overview the characterization of extracellular cellulase components of Microbispora bispora were completed by routine purification and enzyme characterization techniques. These basic results confirmed that the fungal cellulase model can be applied to a procaryotic cellulase system. Endoglucanase and cellobiohydrolase components were purified by ion exchange and gel filtration chromatography, and when recombined, these purified components exhibited synergism during attack on dewaxed cotton and Avicel.

The activities of purified cellobiohydrolase isozymes were further characterized using cellulo-oligosaccharides as substrates. Although these enzymes liberated cellobiose as the sole product from activity toward cellulose, equal molar amounts of cellobiose and cellotriose were produced from the hydrolysis of cellohexaose. These activities are interpreted within the context of similar reports with cellobiohydrolases from other micro-organisms, and may be the result of the fundamental nature of the interaction of insoluble cellulose with all cellobiohydrolases final report (Bartley, 1986).

A description of methodology which was developed to detect and differentiate exoglucanase and endoglucanase enzymes in polyacrylamide gels was published (Bartley et al., 1984). The differentiation was based on a comparison of staining for endoglucanase (carboxymethylcellulose as substrate) and general beta-1,4-glucanase (reduced cellulo-oligosaccharide mixture as substrate) activities. This methodology proved to be valuable during the course of the purification of the cellulase components.

Initiation and characterization of cellulase genes from Microbispora bispora via cloning was begun.

Chromosomal DNA Preparation

Chromosomal DNA from Microbispora bispora was prepared via slight modifications of the technique of Collmer and Wilson (1983), suggestions from U. Priefer (Universitat Bielefeld, Bielefeld, F.R.G.) and our own modification.

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Thus:

Protoplasts can be formed by several cycles of freezing and thawing the cells followed by treatment with lysozyme (10-15 g/ml). Diethylpyrocarbonate is added to inactivate restriction enzymes. The cells are lysed with SDS and RNase added to 50 g/ml. Proteinase K is then added to 50 g/ml. The cell membranes and proteins are removed by successive extractions with phenol and chloroform. The chromosomal DNA is precipitated with sodium acetate and ethanol.

The DNA preparations were used in the cosmid gene banks methodology - see below.

Cosmid vectors

We chose to work with cosmid pSUP106 (Simon et al., 1983a/b), as it offers several advantages in cloning that it contains a broad host range origin of replication (RSF1010), resistance genes for chloramphenicol and tetracycline, the RP4 mobilization site, and can package 20-40kb of foreign DNA. Zymomonas mobilis (see IV) is sensitive to both chloramphenicol and tetracycline (Stokes et al., 1983a) and will maintain plasmids containing the RSF1010 origin of replication (Eveleigh et al., 1983). Therefore, the use of this cosmid should facilitate us to cloning of M. bispora DNA in E. coli, screen recombinants for cellulolytic activity and mobilize the positive recombinants into Z. mobilis. Positive recombinants in Z. mobilis antibiotic resistance traits, will be selected via the expresion of M. bispora cellulase genes thus permitting study of Z. mobilis. Cosmic gene banks can now be constructed using pSUP106 and M. bispora chromosomal DNA. As shown in figure 2, cosmid "arms" are used to prevent polycosmid formation. The chromosomal DNA is partially digested with Sau 3A and ligated to the arms. The recombinant cosmids are then packaged in vitro in a phage and used to infect E. coli where screening for cellulolytic clones will take place.

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III. Other Cellulases

III.A. E. coli cellobiase

As cloning and co-ordinate expression of genes of the total cellulase
system is complex, the use of strains of E. coli that synthesize any of these
genes would be most advantageous. Thus if E. coli produced cellobiase, it would
not be necessary to clone further foreign cellobiase genes into it. In this
vein, we have obtained strains of E. coli that are capable of growth on
cellobiose and other strains that are capable of growth on aryl-glucosides,
arbutin and salicin. These strains are being characterized. e.g., cellobiase is
active against methyl-umbelliferyl-beta-D-glucoside yielding a characteristic
fluorescent aglycone that can be visualized under ultraviolet light. The
cellobiose fermentation can be detected by colorimetric changes on McConkey Agar
with cellobiose, as well as by growth on cellobiose as a sole source of carbon.
These colorimetric reactions are helpful in screening for the cellobiose
utilization genes once they are cloned on pULB113 and in subsequent subcloning.

We obtained E. coli strains MK91 and MK94 from B. Hall. These strains of
E. coli are capable of growth on cellobiose (MK91 and MK94) and the aryl-
glucosides arbutin and salicin (MK94) to another vector and them mobilized to Z.
mobilis. We then showed that the cellobiase function of E. coli MK91 is active
against methyl-umbelliferyl-beta-D-glucoside yielding a characteristic fluorescent
aglycone that can be visualized under ultraviolet light. The E. coli strains
that synthesize cellobiase are most useful, as it is not necessary to first
clone a foreign cellobiase gene into E. coli. The colorimetric reactions
demonstrated will be helpful in screening for the cellobiose utilization genes
once they are cloned on pULB113 and in subsequent subcloning (see Vectors under
Zymomonas below). This approach is well worth further study.

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III.B. Pseudomonas Cellulase

In bacteria, several enzymes can be arranged in a "single" genetic operational unit, the operon, while in eukaryotes (fungi) the control of each enzyme is discreet. We are therefore studying further bacterial cellulases including those from M. bispora but also from cellulolytic Pseudomonas, with the objective of characterizing a cellulase operon and inserting it via recombinant DNA protocols into Zymomonas to gain a fully functional cellulolytic ethanolgen.

The protocols for cloning Pseudomonas cellulase into Zymomonas have been clarified. In an initial transformation step, the intermediate host E. coli S17-1 was shown to be sensitive to $CaCl_2$, but efficient transformation has been regained using $RbCl_2$ with MOPS buffer. Of several Pseudomonds evaluated for conjugation efficiency with E. coli S17-1, one strain PF41 is greatly superior. Screening of recombinants (1000) is underway to select for cellulase containing clones. The vehicle used in these studies pSUP104 has been shown to stably replicate in Z. mobilis. Focus is on endoglucanase. An endoglucanase chromosomal gene from the cellulolytic Pseudomonas fluorescens var. cellulosa (NCIB 10462) was cloned in Escherichia coli. Chromosomal DNA was partially digested with the restriction enzyme EcoRI and ligated into the broad host-range, mobilizable plasmid pSUP104 that had been linearized with the same enzyme. After transformation of E. coli, an endoglucanase-positive clone was detected in situ by use of the Congo-red assay procedure. The endoglucanase gene on the recombinant plasmid pRUCL100 was expressed in the non-cellulolytic Pseudomonas fluorescens PF41. The DNA fragment carrying the gene was transferred to the plasmid pBR322, generating plasmids pRUCL150 and pRUCL151, and its restriction map was derived (Lejeune et al., 1986). Mr. Lejeune is further analyzing the Pseudomonas cellulase system for his Ph.D. at the University of Louvain-la Neuve, Belgium.

IV. Genetic Studies with Zymomonas mobilis

Conversion of biomass to ethanol is best achieved via fermentation. Unfortunately cellulolytic ethanologens (e.g., Clostridium thermocellum) produce relatively low concentrations of ethanol, while in contrast ethanol tolerant (10-12%) microbes such as yeast and Zymomonas cannot degrade cellulose. Our approach has been to enhance the substrate range of Zymomonas to permit use of cellulose by insertion of cellulase genes into the bacterium via recombinant DNA protocols. This should reduce the overall cost of ethanol production by combining the cellulolytic and fermentation stages into a single process.

As Zymomonas is virtually unknown genetically, it was necessary to develop a series of basic genetic methodologies with this bacterium. Conjugation has been the major approach as no viruses attacking it are known and thus no transfection approaches possible. Attempts at transformation has previously proved negative. A variety of genetic protocols were explored.

IV.A. Cloning of Carbohydrate Utilization Genes in Z. mobilis

IV.A (1). Plasmid Transfer to Zymomonas

Expression of the lac Y (lactose transport) and lac Z (beta-galactosidase) has been accomplished and reported previously. Several approaches were used enhance the level of expression of these genes, e.g., by mutation of the regulatory site of the lactose operon, using ultraviolet light and selective conditions to create a mutation allowing for higher levels of expression. None of these methods yielded marked increases in enzyme level. Hence, we considered other options.

IV.A (2). Cloning of a <u>Zymomonas</u> <u>mobilis</u> Plasmid Origin of Vegetative
Replication:

We attempted to clone a <u>Zymomonas</u> <u>mobilis</u> plasmid origin of replication as
the basis for engineering a cloning vehicle for use in <u>Z.</u> <u>mobilis</u> and
<u>Escherichia</u> <u>coli</u>. The <u>Z.</u> <u>mobilis</u> plasmids are stably maintained in the
presence of Inc PI plasmids (e.g., Rsf11010 derivatives such as pKT230 cm^r) and
Inc. FII plasmids (e.g., R1) (Dally, 1983; Eveleigh et al., 1983; Stokes et
al., 1983a/b). A cloning vehicle derived from a <u>Z.</u> <u>mobilis</u> plasmid would
therefore be stably maintained in the presence of the Inc PI and Inc FII
plasmids mentioned above. Such a vector will enable us to develop multiple
plasmid systems. A multiple plasmid system will allow us to clone individual
genes involved in cellulose degradation on different plasmids. They can be
maintained together in the same cell. This approach facilitates study of the
operon structure of genes involved in the cellulose degradation pathway as well
as expression of individual genes of the pathyway.

We are pursuing two methods of cloning a <u>Z.</u> <u>mobilis</u> plasmid origin of
replication. See a and b.

(a) <u>Z.</u> <u>mobilis</u> is sensitive only to a few antibiotics such as chloram-
phenicol and tetracycline. We have prepared a fragment of plasmid pBR325 (Ap^r,
Cm^r Tc^r) by restriction enzyme digestion with the enzymes Pst I and Ava I. Two
fragments were produced by this digestion. The larger fragment contains the
carboxyterminal end of the B-lactamase gene, the chloramphenicol resistance
gene, and the tetracycline resistance gene. The smaller fragment contains the
N-terminal end of the B-lactamase gene and all of the plasmid replication
functions. The fragments were made blunt-ended by treatment with S1 nuclease
or DNA Polymerase I. The two fragments were then separated by gel
electrophoresis. The larger fragment was isolated from gel by electroelution.

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We are attempting to ligate the larger fragment (containing

chloramphenicol and tetracycline resistance genes) to fragments of Z. mobilis

plasmids produced by partial digestion with the enzyme Hae III. Ligation to a

Z. mobilis plasmid fragment containing an origin of replication will enable us

to derive a cloning vehicle with two antibiotic resistance markers useful in Z.

mobilis.

(b) We are in the process of deriving a chloramphenicol resistance gene

fragment from plasmid pHDSG415 (Hashimoto-Goto et al., 1981). This gene is

contained on a 1.3 Kb Hae II fragment. Ligations against partial Hae II

digests of Z. mobilis plasmids are being performed. Due to the fact that Hae II

produces sticky-ends this method should be easier than method a. However

cloning vehicles derived this way will contain only one antibiotic resistance

and will have to be engineered further to introduce other selectable markers.

(c) Results with approaches (a) and (b) continued to be frustrating.

Hence we also have initiated a task to isolate Zymomonas promoters.

IV.A (3). Transformation of Zymomonas mobilis. (M. Yablonsky)

We have achieved a degree of direct transformation of Zymomonas mobilis

CP4 plasmids pKT230 Cm^r (11 Kb) and RP1::Tn951 (73Kb). Transformation was

achieved by growing Z. mobilis CP4 to late phase at 28°C in 2% glucose, 2%

yeast extract, subculturing 1 ml of this culture into 100 ml of the same medium

and allowing the cells to grow at 28°C for 14 hrs. Ninety ml of culture is

pelleted, resuspended in 40 ml of cold 150 mM Mg C1 and held at 4°C for 6 hrs.

Cells, 0.1 ml, and 0.001M Tris-Hcl, 0.001 M EDTA, pH 8.0, 0.02 ml containing 3-

5 ug of plasmid DNA is mixed and held at 4°C for 1 hr. The mixture of cells

and DNA are heat-shocked at 42°C for 2 min, 1 ml of 2% glucose, 2% yeast

extract at 28°C is added and the mixture is incubated anaerobically at 28°C for

6 hrs. Transformed cells are selected by plating appropriate serial dilutions

of the mixture onto medium containing 2% glucose, 2% yeast extract and the appropriate antibiotics. Transformed cells are purified on the same medium. The method has been gradually improved and higher rates of transformation have been obtained (1.7×10^3 transformants/ug DNA).

The importance of direct transformation of Z. mobilis with plasmid DNA cannot be overstated. Up to this point the introduction of foreign plasmids and cloning vectors into Z. mobilis has been dependent on self-transmissability of the foreign plasmids or the presence of mobility sites on cloning vectors which allow them to be conjugally transferred from a donor organism to Z. mobilis will allow us to utilize plasmids and cloning vectors which do not possess the above mentioned transmissability and mobility properties. Also, selection of transformed Z. mobilis is much clearer than selection of transconjugants since there is no donor organism to eliminate during the selection process.

The rates achieved to date do not approach the transformation rate of E. coli (10^6 transformants/ug DNA). Therefore we will continue to use E. coli as the primary host for recombinant plasmids. However, non-mobilizable recombinant plasmids may now be introduced into Z. mobilis via the transformation protocol.

IV.A (4). Antibiotic sensitive strains:

Plasmid transfer can be accomplished through conjugation and transformation. Conjugational transfer systems are now well developed using self-transmissable, and also helper plasmids for mediating the transfer of other plasmids. One hindrance to the procedure has been the lack of the use of multi-cloning site vectors due to the inherent antibiotic resistance of Zymomonas. Antibiotic sensitive strains were therefore developed and these extend the usefulness of the multi-antibiotic resistant vectors. In all of the

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above instances, expression of antibiotic resistance in <u>Zymomonas</u> has been obtained following conjugation but to date expression of other genes has not been detected. Subsequent mutation has not changed this state (Buchholz and Eveleigh, 1986).

Plasmidless Strains of <u>Zymomonas</u> (A.A., M.D.)

A plasmidless strain of <u>Z. mobilis</u> would considerably aid recombinant DNA studies by facilitating the identification of strains that have taken up plasmids. Attempts to "cure" <u>Z. mobilis</u> strains by use of high temperature, sodium dodecyl sulfate and nalidixic acid and mitomycin C were unsuccessful. The approach is now to isolate plasmidless strains via continuous culture under phosphate limitation. Plasmid loss in the population is being monitored via analysis of retention of antibiotic resistance (tetracycline) associated with the RP4 plasmid. To date, no method has yielded strains with reduced plasmid content.

IV.A (5). <u>Extractive Fermentation for Ethanol Production</u>.

<u>Z. mobilis</u> was grown in the presence of Isopar M. tridecyl alcohol (IMTA) (50:50, v/v) (received from D.W. Tedder, Georgia Tech.) to assess the possibility of combined fermentation extraction. IMTA did not appear toxic to <u>Z. mobilis</u> over a 4-20% range, though enhanced ethanol productivities were not obtained. However, further testing in batch culture did not give enhanced ethanol production. The methodology course bear further examination under more controlled fermentor conditions.

SUMMARY

IA (1). Cellulase production by <u>T. reesei</u> cells immobilized Celite has yielded high enzyme productivities with average production of 175 filter paper (fpu) units/1/hr (high yields of 270 fpu/1/hr). Several nutrient feeding ranges have been studied, and maximum cell loading of 107 g/1 fermentor with 2g cell/g Celite have been achieved. Reduction in carbon supply perturbed the steady state population resulting in lower cell populations but transiently higher cellulase productivities. These latter may simply be artefactual and a result of some cell lysis. Cellulase production by mycelial pellets was compared to that using Celite immobilized cells. Pellets (5-8 mm diam.) formed readily and cellulase production was followed for roughly three weeks. Yields averaged 50 fpu.1.hr and is probably a result of lower cell loadings (e.g., 70 g/1 c.f. 100 g/1 of Celite immobilized cells).

IA (2). Sporulation: A Celite-immobilized culture was subjected to stepwise decreasing carbon:nitrogen (C:N) ratios from 8:1 (routine cellulase production) to 22:1, while maintaining a constant lactose feed. Cellulase production became reduced under these conditions but sporulation became marked at the 12:1 feedrate. Phialospore counts reached 2×10^7/ml effluent and was equivalent to 2×10^9 spores.1.hr. Over 90% of the phialospores were viable. A direct descending lactose gradient resulted in thinner less branched hyphae, and large inter-calary thick walled chamydospores were also formed. These findings can have application in projects utilizing massive spore inocula, e.g. control of plant pathogens.

IA (3). Glycosylation: <u>Trichoderma</u> cellulase components that have been de-glycosylated via enzymatic cleavage (Endo-H treatment) or by synthesis in the presence of tunicamycin, continue to show activity equivalent to that of the

fully glycosylated enzyme. No change in temperature stability of the deglycosylated

enzymes was noted. These results are considered promising in relation to

prokaryotic processing of T. reesei RNA and with regard to the apparent lack of

a need for glycosylation in order to maintain enzyme activity.   (For final

report see K. Murphy-Holland, Ph.D. thesis 1985).

II.   The characterization of extracellular cellulase components of

Microbispora bispora were completed by routine purification and enzyme

characterization techniques. These basic results confirmed that the fungal

cellulase model can be applied to a procaryotic cellulase system.

Endoglucanase and cellobiohydrolase components were purified by ion exchange

and gel filtration chromatography, and when recombined, these purified

components exhibited synergism during attack on dewaxed cotton and Avicel.

The activities of purified cellobiohydrolase isozymes were further

characterized using cellulo-oligosaccharides as substrates. Although these

enzymes liberated cellobiose as the sole product from activity toward

cellulose, equal molar amounts of cellobiose and cellotriose were produced from

the hydrolysis of cellohexaose. These activities are interpreted within the

context of similar reports with cellobiohydrolases from other micro-organisms,

and may be the result of the fundamental nature of the interaction of insoluble

cellulose with all cellobiohydrolases final report (Bartley, 1986).

IIIA. The E. coli strains that synthesize cellobiase are most useful, as it

is not necessary to first clone a foreign cellobiase gene into E. coli. The

colorimetric reactions demonstrated will be helpful in screening for the

cellobiose utilization genes once they are cloned on pULB113 and in subsequent

subcloning (see Vectors under Zymomonas below).

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IIIB.   An endoglucanase chromosomal gene from the cellulolytic <u>Pseudomonas</u>
<u>fluorescens</u> var. <u>cellulosa</u> (NCIB 10462) was cloned in <u>Escherichia</u> <u>coli</u>.
Chromosomal DNA was partially digested with the restriction enzyme EcoRI and
ligated into the broad host-range, mobilizable plasmid pSUP104 that had been
linearized with the same enzyme.   After transformation of <u>E.</u> <u>coli</u>, an
endoglucanase-positive clone was detected in situ by use of the Congo-red assay
procedure.   The endoglucanase gene on the recombinant plasmid pRUCL100 was
expressed in the non-cellulolytic <u>Pseudomonas</u> <u>fluorescens</u> PF41.   The DNA frag-
ment carrying the gene was transferred to the plasmid pBR322, generating
plasmids pRUCL150 and pRUCL151, and its restriction map was derived.

IV.   The development of a basic genetic system for studying the ethanologen was
addressed.   Following insertion of the lac (beta-galactosidase) operon into
<u>Z.</u> <u>mobilis</u>, subsequent and mutation failed to raise beta-galactosidase levels.
In order to gain enhanced enzyme production, fundamental genetic protocols were
considered including the isolation of <u>Zymomonas</u> origins of replication and
promoters.   Studies on developing a <u>Zymomonas</u> transformation were partially
successful.   Antibtiotic sensitive mutants were isolated and have proved useful
in facilitating plasmid transfer to <u>Zymomonas</u> based on selection of inheritance
of antibiotic resistance.

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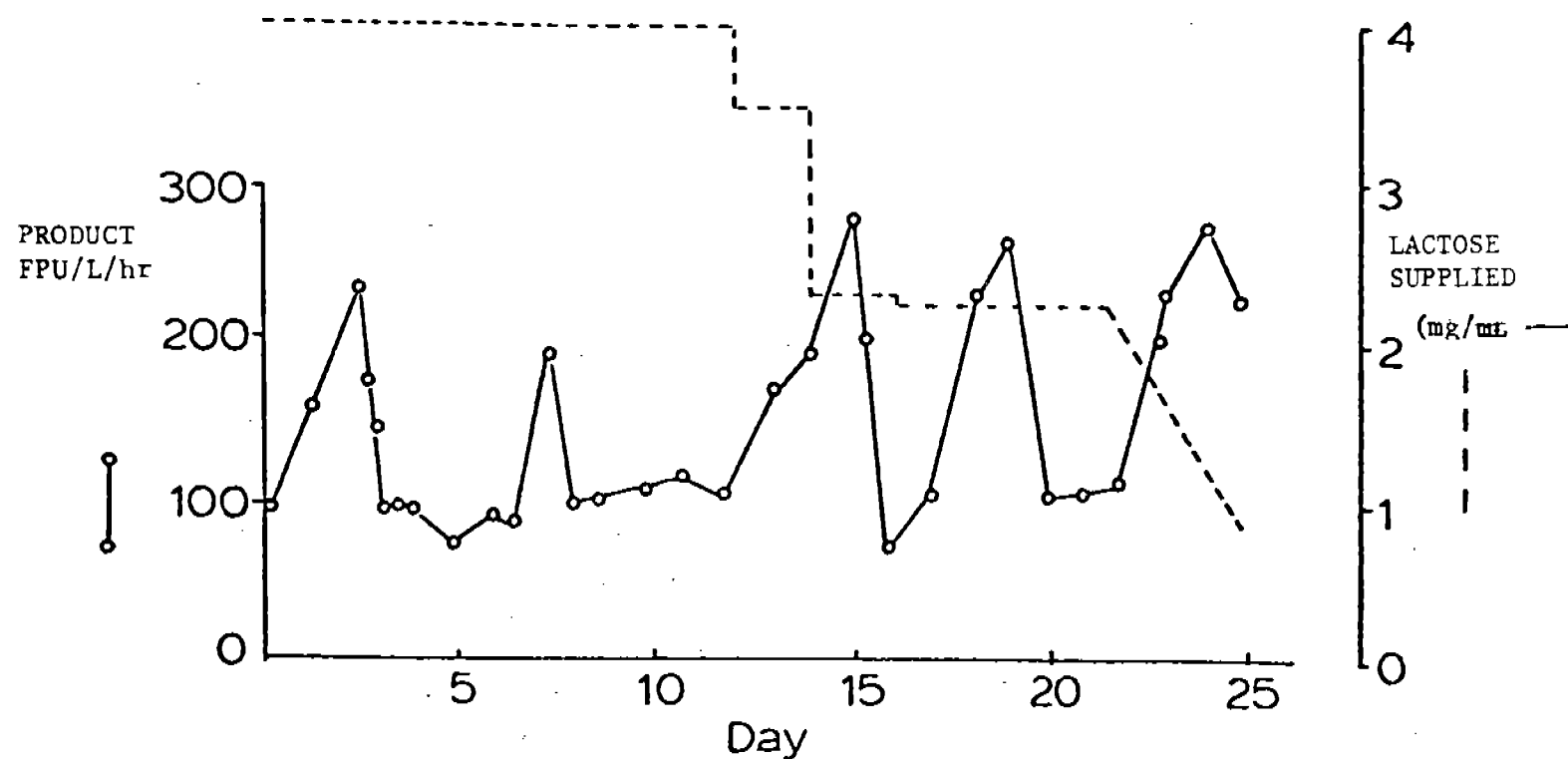
Figure 1

FIG. 1A- Cellulase production during carbon starvation.
　　　　Conditions: Celite-60 g/L; Vogels medium, Dilution rate 0.1;
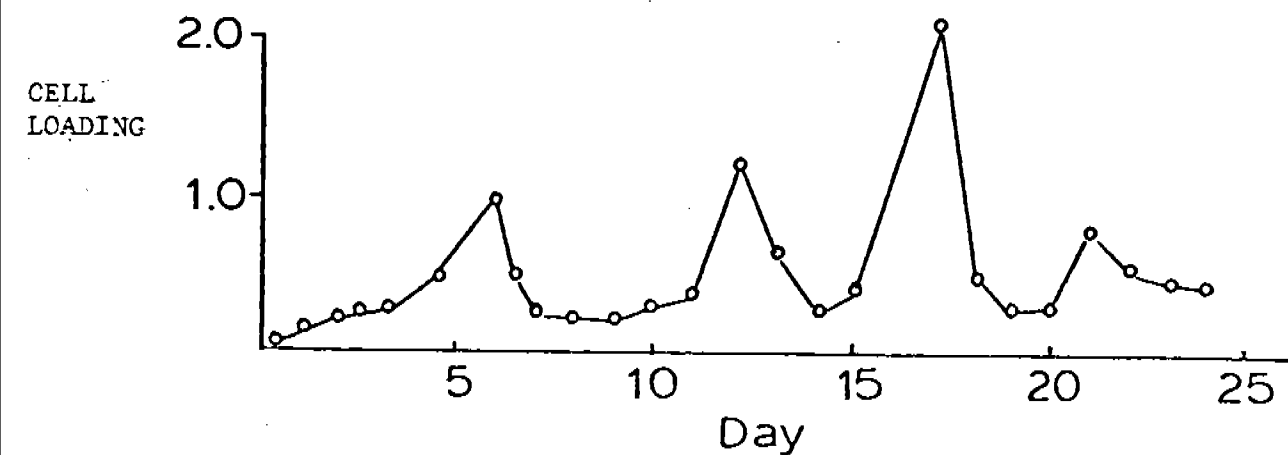　　　　Aeration 2 vvm.

FIG. 1B- Effect of carbon starvation on cell loading (g cell/g support).
　　　　Conditions - as above.

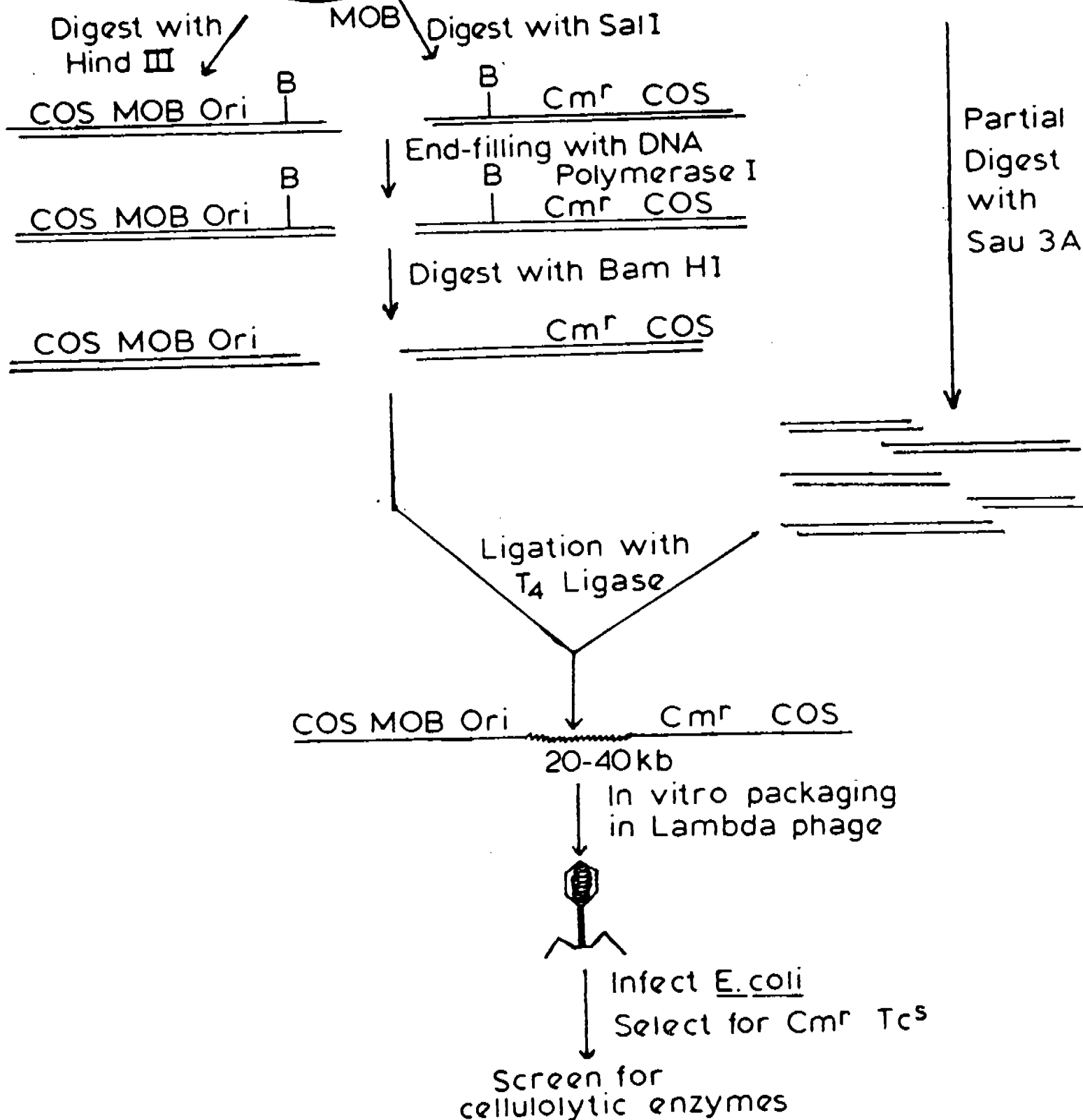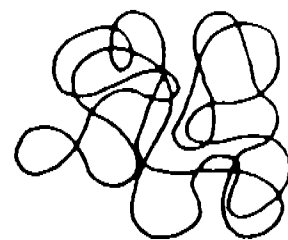Figure 2.   Cloning of M. bispora cellulase enzymes

TABLE I. Plasmids

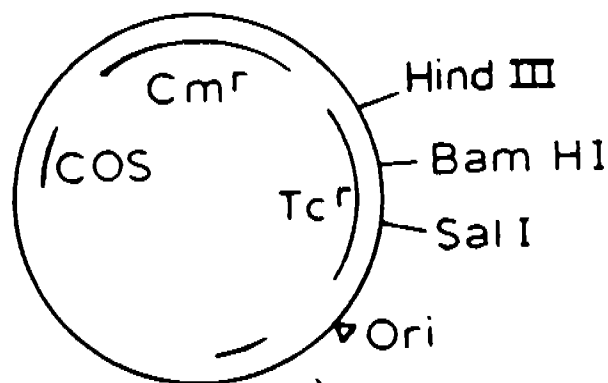| Plasmids	Host	Source	Reference
pRK290 tet^r,mob^t	HB101	Helinski, D.	pers. comm.
pRK291 tet^r	HB101	Helinski, D.	pers. comm.
pRK292 tet^r	HB101	Helinski, D.	pers. comm.
pRK293 tet^r	HB101	Helinski, D.	pers. comm.
pRK404 tet^r	HB101	Helinski, D.	pers. comm.
pULB113 tet^r, Kan^r. Ap^r, Tra^+, Chr^+	MXR	Lejeune, A.	pers. comm.
pVK100 tet_r, Kan_r Cos	HB101	Nester, E. W.	pers. comm.
pVK102 tet_r, Kan_r, Cos	HB101	Nester, E. W.	pers. comm.
RP4 tet_r, Kan_r, Ap_r	J53	Lejeune, A.	pers. comm.
pMC1403 Ap_r, Lac Z, Y, A	Mc1061	Sofer, W.	see Picataggio thesis
pSKS105 Ap_r Lac IPOZYA	YMC9	Picataggio, S.	see Picataggio thesis
pSKS106 Ap_r Lac IPOZYA	YMC9	Picataggio, S.	see Picataggio thesis
pSKS107 Ap_r Lac IPOZYA	YMC9	Picataggio, S.	see Picataggio thesis

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